The development of the gut microbiota in rainbow trout (Oncorhynchus mykiss) is affected by first feeding and diet type

Ingerslev, Hans-Christian; von Gersdorff Jørgensen, L.; Strube, Mikael Lenz; Larsen, N.; Dalsgaard, Inger; Boye, Mette; Madsen, Lone

Published in:
Aquaculture

Link to article, DOI:
10.1016/j.aquaculture.2013.12.032

Publication date:
2014

The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type

H.-C. Ingerslev, L. von Gersdorff Jørgensen, M. Lenz Strube, N. Larsen, I. Dalsgaard, M. Boye, L. Madsen

PII: S0044-8486(13)00687-X
DOI: doi: 10.1016/j.aquaculture.2013.12.032
Reference: AQUA 630978

To appear in: *Aquaculture*

Received date: 30 October 2013
Revised date: 13 December 2013
Accepted date: 17 December 2013

Please cite this article as: Ingerslev, H.-C., von Gersdorff Jørgensen, L., Strube, M. Lenz, Larsen, N., Dalsgaard, I., Boye, M., Madsen, L., The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type, *Aquaculture* (2014), doi: 10.1016/j.aquaculture.2013.12.032

The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type

H.-C. Ingerslev[1]*, L. von Gersdorff Jørgensen[2], M. Lenz Strube[1], N. Larsen[3], I. Dalsgaard[1], M. Boye[1] & L. Madsen[1]

[1]National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark. *E-mail: hain@vet.dtu.dk. Phone: +4535886236.

[2]Laboratory of Aquatic Pathobiology, Section of Biomedicine, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 7, DK-1870 Frederiksberg C, Denmark.

[3]Danish Genome Institute, Skt. Lucas Kirkeplads 8, DK-8000 Aarhus C, Denmark.

Abstract

An influence of the intestinal microbiota in connection to first-feeding of rainbow trout (*Oncorhynchus mykiss*) fry was demonstrated using Illumina HiSeq sequencing. The period from the end of yolk-sac feeding and until seven weeks post first-feeding was examined after administration of either a marine- or plant based diet with or without the probiont *Pediococcus acidilactici*. Before first feeding the main part of the sequence reads grouped to the genus *Sediminibacterium* probably originating from the surrounding water. The microbial abundance and diversity increased after first-feeding and the microbiota then changed towards Firmicutes phylum dominance for plant based fed fish and towards dominance of phylum Proteobacteria for the marine fed fish. After first-feeding, there were significantly higher abundances of *Streptococcus*, *Leuconostoc* and *Weissella* in fish fed the plant-based diet. The microbiota clustered separately according to the diet type, but only minor effects were seen from the probiont when using PCA-analysis. The constitutive transcription level of most examined immune genes increased during the ontogenic shift, but the results could not explain the differences in the composition of the

microbiota dependent on diet treatment after first-feeding. The results suggest that the intestine of rainbow trout is colonised at an early state, but is guided in new and different directions dependent on the diet type.

1. Introduction

Bacteria are found on outer surfaces of fish such as the gills, the skin and on inner surfaces like those of the gastrointestinal tract. Most of these bacteria are usually harmless and symbiotic and may reflect the microbiota from the surroundings (Hansen and Olafsen, 1999). During the very initial developmental phase of all bony fish species the nutrition comes from a yolk sac only, which originates from the egg. At that time the intestine is an undifferentiated straight tube, but develops and matures simultaneously with the consumption of the yolk sac and initiation of feeding from external food sources. Prior to initiation of first feeding the gastrointestinal system is in contact with the surrounding water due to physical passage through the digestive tract (Rombout et al., 2011; Hamlin et al., 2000). In larvae of turbot (*Scophthalmus maximus*) bacteria could be detected already 1 day post hatch (Ringo et al., 1996). In fish the intestinal microbiota is known to influence several parameters like stimulation of epithelial proliferation, the degree of nutrient harvest, the physiological development as well as the intestinal immune responses (Semova et al., 2012; Bates et al., 2006; Falkow, 2006; Salinas et al., 2005; Rawls et al., 2004). Most of the characterised bacteria in these studies belonged to the β- and γ-subclasses of the phylum Proteobacteria and a few bacteria from phylum Actinobacteria were also found. Different genera of lactic acid bacteria (phylum Firmicutes) have also been described as a normal part of the intestinal microbiota of several fish species (Roeselers et al., 2011; Hovda et al., 2007; Ringo and Gatesoupe, 1998). Immunologically, transcription of innate immune genes such as Saa1 and C3 is negatively influenced by the lack of gut bacteria in germ-free zebrafish (*Danio rerio*) (Rawls et al., 2004).

Application of probiotic bacterial strains may on the other hand positively stimulate the immune system and directly influence the surrounding microbial community in the gut (Ferguson et al., 2010; Arijo et al., 2008; Balcazar et al., 2007). The colonisation of the intestine in connection to feeding and the impact of the diet on the microbiota in general in fish has to date mainly been examined using traditional techniques like Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (PCR-DGGE), Fluorescent *In Situ* Hybridization (FISH) and plating of cultivable species (Hovda et al., 2007; Kim et al., 2007; Romero and Navarrete, 2006; Bates et al., 2006; Huber et al., 2004; Ringo et al., 1998). Usually only a low fraction down to about 1% of the bacteria from the intestine of fish is cultivable, which makes molecular techniques advantageous when examining complex microbial communities (Navarrete et al., 2010; Romero et al., 2006). In some mammalian species like humans and pig this fraction may, however, be much larger (Flint et al., 2007). In recent years advanced next-generation sequencing techniques of bacterial DNA have enabled high-throughput sequencing of entire microbial communities (Mardis, 2008). A few studies have examined the gut microbiota from zebrafish and rainbow trout (*Oncorhynchus mykiss*) using pyrosequencing (Desai et al., 2012; Roeselers et al., 2011).

Due to the growing production of fish in the worldwide aquaculture industry oils and proteins from plants are being applied in various amounts as supplements for marine oils and fish meal to some types of carnivorous fish feed. Earlier studies have indicated that the type and origin of the feed ingredients (marine versus plant origin) may influence the intestinal epithelial structures and the local immune status in the gut (Mourente et al., 2007; Caballero et al., 2002; Bell et al., 1996). In general, probably mediated via modulation of the cell membrane, fatty acids modulate cytokine expression, phagocytic- and NK-cell activity (de Pablo and de Cienfuegos, 2000). In rainbow trout a diet containing proteins from vegetables such as pea and soy generally led to a higher amount of bacteria belonging to the phylum Firmicutes relative to the phylum Proteobacteria compared to a

fish meal based diet (Desai et al., 2012). Other plant diets containing lupin were shown to affect the microbial diversity by decreasing the taxon diversity in goldfish (*Carassius auratus*) (Silva et al., 2011).

In the present study the gut microbiota in rainbow trout was examined on the larval stage from one day before first feeding and until 49 days post first feeding using Illumina HiSeq high-throughput sequencing of the partial 16S rRNA gene. Two different diets were evaluated in order to examine how the initial gut microbiota changes from the very first time the fish gets external nutrition. The diets included were i) a marine diet containing fish meal and fish oil and ii) a plant-based diet where the fish oil was completely replaced by rape-seed oil and a part of the fish meal was further replaced by pea meal. Further, each diet type was administered to the fish either surface coated with the commercial probiotic Bactocell® (*Pediococcus acidilactici*) or without in order to investigate whether the probiont could influence the composition of the gut microbiota. To study whether the different diets influenced the intestinal immune status of the fish and to correlate the immune response with the microbial community pattern the transcription level of a panel of the following immune genes were measured by qRT-PCR: MBL1-3, iNOS, C3, C5, IL-1β, CD4, CD8, FOXP3a, FOXP3b and membrane bound IgT and IgM.

2. Materials & methods

2.1. Fish, rearing conditions and diets

Fertilized eggs (n = 13,000) at the eyed egg stage from rainbow trout were brought to hatching facilities at DTU Aqua, Technical University of Denmark (Hirtshals, Denmark) from a conventional fish farm in Jutland, Denmark on August 3rd 2011. After arrival the eggs were disinfected in iodophore (100 ppm) for 15 min. followed by washing with clean water (Actomar K30, Aqualogistik GmbH, Möhnesee-Wippringsen, Germany). Hatching occurred around August 10 and

the fry was two weeks post hatch transferred to new, rearing facilities at the research facilities of Biomar A/S in Hirtshals, Denmark. The fish larvae were equally split into 8 circular tanks with a volume of 100 L each. The fish tanks were supplied by re-circulating fresh water connected to a bio-filter common for all tanks, temperature control and exposure of the water to UV-light. Each tank was separately supplied by oxygen and the saturation was between 100-150% throughout the entire experiment. The temperature of the water was measured daily. Initially the temperature was 10°C, but gradually elevated to 12-13°C during the following week. The average temperature (°C ± SD) at the Biomar rearing facilities was 12.4 ± 1.0°C. The fish were exposed to a light regime of 12 hours of light and 12 hours of darkness. First-feeding (f.f.) was initiated about three weeks after hatching. For the first two weeks after f.f. the fish were fed according to appetite, but thereafter at a daily rate corresponding to about 2.5% of bodyweight. The 8 tanks were divided into four different dietary groups giving two tanks per diet group. The specific diets were applied to the fish throughout the experiment from f.f. and until termination. Diet A (INICIO, Biomar A/S, Brande, Denmark) was commercial fish feed and contained fish meal and fish oil from marine sources. Diet B was INICIO Plus (Biomar A/S, Brande, Denmark) and similar to INICIO except that it contained a probiotic bacterium, *Pediococcus acidilactici* provided by Lallemand Animal Nutrition (www.lallemandanimalnutrition.com) as Bactocell®. The amount of *P. acidilactici* in the feed was the same as to the one used in the commercial INICIO Plus and about $7x10^5$ cfu/g. Diet C was custom-made and contained rape seed oil, which completely replaced the fish oil used in preparation of diet A and B. Additionally, diet C contained fish meal, but 10% of the fish meal used in diet A and B was replaced by pea meal in diet C. Diet D was similar to diet C, but contained *P. acidilactici* in the same amount as for diet B. All four diets contained the same energy distribution (% ± SD); 56.5 ± 1.2 of protein, 15.2 ± 1.7 of fat, 6.3 ± 0.4 of water and 11.5 ± 0.6 of ash.

All procedures were conducted in accordance with the regulations set forward by the Danish Ministry of Justice and Animal Protection committees by Danish Animal Experiments Inspectorate permit 2012-15-2934-00573.

2.2. Sampling for microbiome analysis

Prior to sampling the fish were killed with an overdose of MS-222 anaesthetics (Sigma-Aldrich, St. Louis, MO, USA) and their weight and length were measured. Samples were taken at three different time points during the experiment; 1 day before f.f. was initiated (equivalent to 19 days after hatch) and then at days 26 and 49 post first-feeding. To test for size differences (weight and length) among the diet groups a one-way ANOVA and Bonferroni was performed using GraphPad prism version 5.00 for Windows (GraphPad software, San Diego CA USA).

Fifty fish were collected for analysis one day before f.f. At days 26 and 49 post f.f., respectively, six fish were sampled from each tank corresponding to 48 fish per sampling point. The complete intestine was sampled using a sterile scalpel and forceps. If present, fecal content was removed by squeezing along the exterior side of the intestine. The tissue was subsequently stored at -20 °C until further analysis.

2.3. Sampling for qRT-PCR

Another subsampling of fish was performed for the qRT-PCR using the procedures as described above. On day 1 before f.f., 10 fish were sampled, while 40 fish (5 fish per tank) were sampled from the same tanks as for the microbiome studies at days 26 and 49 post f.f. At all sampling points the entire intestine was excised and immediately placed in RNAlater^{TM} (Sigma-Aldrich) at 4 ºC for 24 hours before they were frozen at -20 ºC.

2.4. Bacterial examination for fish pathogens

Fish were killed and examined for bacterial pathogens to ensure that no fish were infected during the experiment. At each sampling point 40 fish in total were sampled, covering 5 fish from each group equivalent to 10 fish from each diet code at days 26 and 49 post f.f.. Samples from brain, kidney and spleen (spleen was not sampled from yolk sac fry) were inoculated directly on tryptone yeast extract salts (TYES) agar (Holt et al., 1993), an agar that supports growth of fish pathogens like *Flavobacterium psychrophilum*, but also *Yersinia ruckeri* and *Aeromonas salmonicida*. The intestine was aseptically removed, squeezed in sterile TYES media, and plated on TYES agar and Blood Agar (BA). The BA plates were incubated at 20°C for up to 7 days and the TYES agar plates at 15°C for up to 14 days and checked for bacterial growth with regular intervals (Dalsgaard and Madsen, 2000).

2.5. DNA extraction and purification for the microbiome analysis

DNA was extracted from the collected intestines for use as PCR template. The intestines of each fish were shaken with 300 µL of lysis buffer provided in the Maxwell® LEV Blood DNA Purification Kit (Promega Corporation, Madison, WI, USA) and one 5 mm stainless steel bead (Qiagen GmbH, Hilden, Germany) was added to the tissue followed by shaking on a Qiagen TissueLyser II (Retsch GmbH, Haan, Germany) for 2 min at 20 Hz. DNA was then extracted on a Maxwell® 16 Research Instrument System using a Maxwell® LEV Blood DNA Purification Kit (Promega Corporation) according to the manufacturer's instructions. The concentration of the DNA was then quantitated on a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

2.6. Quantification of 16S DNA from intestinal samples

The amount of 16S DNA in the samples from the intestines was measured by qPCR for each fish in order to determine the bacterial load at every sampling point. Isolated DNA samples were diluted to 40 ng/µl for normalisation and used as template in the qPCR reaction. The PCR reactions were setup in 25 µl reaction volumes containing 1 µl of the universal primers (Pedersen et al., 2013) 804f and 926r (10 µM), 0.5 µl of $MgCl_2$ (25 mM), 12.5 µl of SYBR® Green Jumpstart™ *Taq* ReadyMix™ (Sigma-Aldrich), 2 µl of DNA template (40 ng/µl) and 9 µl of nuclease free water. The PCR reactions were carried out in a Rotor-Gene Q real-time PCR instrument (Qiagen). Reaction times and cycling conditions were 94°C for 2 min, 40 cycles of 94°C for 30 s and 60°C for 1 min. The run was further terminated by a melting curve analysis. Obtained C_t-values were normalised against the amount of DNA used for the PCR reactions and plotted after verification that the primers amplified with an efficiency of about 100% using a standard curve, which is equivalent to a doubling of the PCR products in the log-linear phase. Statistical analysis was performed by Kruskal-Wallis test and Dunns post-test using GraphPad prism version 5.00 for Windows (GraphPad software, San Diego CA USA). *p*-values < 0.05 were considered significant.

2.7. 16S rDNA PCR

PCR was performed targeting the V5 region of the bacterial 16S rRNA gene from bacteria present in the intestinal tissue. The PCR was performed using the universal primers 804f (5'-GGATTAGATACC CNGGTAGTC-3') and 926r (5'-CCGTCAATTCCTTTRAGTTT-3') (Sigma-Aldrich). Both primers were 5'-barcode tagged (6-nt) and each specific barcode were assigned a specific DNA sample. The reaction was carried out in 50 µl reactions containing 5 µl of 5 x Goldtaq buffer (Applied Biosystems, Branchburg, NJ, USA), 1µl of each primer (20 µM), 2 µl of 10 mM dNTP, 4 µl of 25 mM $MgCl_2$, 0.5 µl of AmpliTaq Gold® polymerase (Applied Biosystems), 35.5 µl of nuclease-free H_2O and 1 µl of DNA template (1000 ng). Reaction times and cycling conditions

were 94°C for 6 min, 30 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 45 s and 72°C for 10 min. The resulting PCR products were then analysed on an Agilent 2100 Bioanalyzer using the Agilent DNA 1000 kit (Agilent Technologies, Waldbronn, Germany) and further pooled in equimolar ratios. The pooled DNA was then purified for primers and detergents using a Qiagen MiniElute PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.8. Sequencing of PCR products and bioinformatics analysis

The DNA was submitted to the National High-throughput DNA Sequencing Centre at Copenhagen University, Denmark, for sequencing on an Illumina HiSeq^{TM} 2000 platform. The obtained 101-bp long reads were analysed using the BION-meta software (for more information about BION-meta and for acquisition of software see http://box.com/bion). In brief, the sequences were initially de-multiplexed according to the primer- and barcode sequences. Then they were cleaned at both ends by removal of bases of a quality less than 96%, which is equivalent to a Phred score of 14. Identical sequences were further clustered and aligned into consensus sequences. Consensus sequences of at least 30 nucleotides in length were mapped into a table according to the individual barcodes. Finally, the consensus sequences were taxonomically classified against the Greengene SSU database using a word length of 8 and a match minimum of 30%. The top one percent of the obtained similarities from the Greengene database was used for taxonomical classification of the consensus sequences. Due to a sequence length of about 100 bases of the 16S rRNA gene the taxonomical classification was performed down to genus level and in some cases only to family level. The number of reads for each barcode were further normalised in order to enable direct statistical calculations of relative abundance of a given bacterium between individuals in the experiment. The resulting microbial profiles were log transformed and statistically analysed in

GraphPad prism version 5.00 for Windows (GraphPad software, San Diego, CA, USA), specifically using one-way ANOVA and Bonferroni post-test to test for differences in microbial community composition between the different diet groups. *p* -values < 0.05 were considered significant. An analysis was carried out at the phylum level and phyla with an overall abundance below 1% of the total amount of reads were not considered for statistical analysis.

2.9. Shannon diversity index (H')

The Shannon diversity index (*H'*) was calculated from the 16S rRNA community profile of each fish to quantify the entropy of the present bacteria (Shannon, 1948). The overall 95% most frequent genera and their relative abundances were chosen for the analysis. The *H'* means and standard variations were further calculated for each diet type. To test whether there was significant difference among treatment groups (diet types) one-way analysis of variance (ANOVA) was performed followed by Bonferroni's Multiple Comparison Test. *p*-values below 0.05 were considered significant.

2.10. Multivariate analysis of bacterial communities

To examine the influence of the ontogenic development and diet type in relation to the microbiota the bacterial communities were analysed by Principal Component Analysis (PCA) using the princomp function and correlation matrix in the open-source tool R (R Core Team, 2013). By using this analysis it could be shown which genera that explained the highest variation in the dataset. For the analysis the relative abundance of the most frequent genera for every individual fish at each sampling date were used. The number of genera included in the analysis was the same as the number of individual fish at the specific sampling date. Each individual fish were afterwards plotted

and the two components explaining the highest degree of variation for each dataset were used for the plotting.

2.11. Reverse transcriptase qPCR

To investigate the transcription level of immune relevant genes in the intestine before and after f.f., RT qPCR analyses using real-time PCR were performed. The following genes were examined: MBL1-3, iNOS, C3, C5, IL-1β, CD4, CD8, FOXP3a, FOXP3b and membrane bound IgT and IgM. RNA purification, cDNA synthesis and real-time qPCR were conducted according to the methods described by Jørgensen et al. (Jorgensen et al., 2008). The Stratagene MX3000P^{TM} real-time PCR system was used and primers and probes are shown in Table 1. The probes used were 5' FAM labelled (Sigma-Aldrich). Negative controls included both a mock reverse transcription reaction (RT minus) and a master mix setup with nuclease free water instead of DNA template. The three housekeeping genes (HKG) 28S ribosomal protein, elongation factor-1α and beta-actin were investigated for stability in transcription level. The HKG with the lowest variation among the C_t-values within every sampling point was selected for normalisation using a t-test ($P < 0.05$).

3. Results

3.1. Fish growth and occurrence of pathogenic bacteria

The sampled fish had a mean weight (g ± SD) and length (cm ± SD) of 0.12 ± 0.01 g and 2.02 ± 0.11 cm at 1 day before f.f., 0.31 ± 0.08 g and 3.3 ± 0.2 cm at day 26 post f.f. and 1.08 ± 0.27 g and 4.66 ± 0.37 cm at day 49 post f.f. (data not shown). There was no statistical difference in the mean size of the fish regarding weight and length between the four diet groups at the sampling points 26 and 49 days post f.f., respectively ($p > 0.05$). The bacterial investigation for fish pathogens showed no presence of pathogenic bacteria at any of the samplings.

3.2. 16S rDNA PCR and sequence data

The sequence files from the Illumina HiSeq® 2000 were analysed using the Bion-Meta software. A total number of 114,882,532 reads were obtained from the sequencing centre. After de-multiplexing according to the sequences of barcodes and primers 90,148,272 sequences were left and these were further 3' and 5' trimmed according to the quality. Sequences below a quality of 96% were sorted out. The number of sequences used for taxonomical classification was then 60,465,201, which was equivalent to an average of 539,867 reads per sample. Of these 99.79% were taxonomically classifiable according to the Greengene SSU database.

3.3. Bacterial load in intestinal tissue samples

The bacterial load in the intestines of sampled fish was determined on basis of the quantity of 16S rDNA relative to the amount of DNA used for the qPCR reaction (Fig. 1). The relative expression of 16 S rDNA in the intestines of the fish one day before f.f. was significantly lower (relative expression ± SD) (0.057 ± 0.027) than for the groups of fish from 26 (0.079 ± 0.029) and 49 days post f.f. (0.074 ± 0.029), respectively ($p < 0.05$).

3.4. Shannon diversity

The Shannon diversity index was calculated for each fish intestine based on the 95% most abundant taxa in the sample, and averaged by diet. As a result, between 100 and 110 bacterial taxa were included in this analysis for each sample (Fig. 2). The *H'* value (mean ± SD) 1 day before f.f. was 2.25 ± 0.42, while it was significantly higher 26 and 49 days post f.f. for all four diets, respectively ($p < 0.05$). There was no significant difference in the *H'* value between the four diets or between the sampling points 26 and 49 days post f.f. ($p > 0.05$).

3.5. Microbial community profiling - phylum level

The bacterial communities of the intestines from all samplings were constituted of a total of 14 different bacterial phyla (Fig. 3, a-c). The overall most abundant 10 phyla at the different sampling points represented between 99.3 and 99.9 % of the entire sequence reads. There was no significant effect of the probiotics on the relative abundance of the different phyla at any of the samplings post f.f. ($p > 0.05$). One day before f.f. the microbiota was dominated by bacteria from phylum Bacteroidetes and constituted (% ± SD) 47.9 % ± 17.2 of the entire reads. The abundance of Bacteroidetes was significantly lower for plant + pro (6.7 % ± 5.5) and plant (7.1 % ± 1.4) groups of fish 26 days post f.f. compared to 1 day before f.f. ($p < 0.001$). Within the sampling point 26 days post f.f. the number of bacteria belonging to the phylum Bacteroidetes was significantly higher in marine fed fish relative to plant fed fish ($p < 0.05$). At 49 days post f.f. Bacteroidetes was significantly lower for all diet groups relative to before f.f. ($p < 0.0001$). The abundance of Bacteroidetes at 49 days post f.f. was overall between 2.1 % and 8.2 %.

Phylum Proteobacteria constituted 26.7 % ± 16.6 of the microbiota before f.f., but was significantly higher for the marine diet groups at 49 days relative to before f.f. ($p < 0.0001$), where it became the dominant phylum and constituted 61.8 % ± 10.6 and 71.3 % ± 13.3 of all reads for the marine + pro and marine group, respectively. The abundance of phylum Proteobacteria did also increase for the plant diet group 49 days post f.f. in comparison to the before f.f., and constituted 45.5 % ± 18.3 of the entire reads ($p < 0.01$).

The abundance of the phylum Firmicutes was 13.7 % ± 12.6 before first feeding, but was significantly higher for the plant fed groups 26 days post f.f. ($p < 0.0001$). The Firmicutes abundance for these groups was 47.0 % ± 18.2 and 41.8 % ± 20.3 for plant + pro and plant fed fish, respectively. Further, Firmicutes abundance for both plant diet groups was significantly higher than

for both marine diets group ($p < 0.05$). The same pattern was seen 49 days post f.f. Here the Firmicutes abundance for both plant diet groups was significantly higher than before f.f. ($p < 0.0001$) and significantly higher than both the marine diet groups 49 days post f.f. ($p < 0.05$). The abundance of Firmicutes for the plant + pro and plant groups 49 days post f.f. were 43.6 % ± 23.1 and 40.3 % ± 20.9, respectively. The Actinobacteria was the fourth most frequent phylum before f.f. and constituted 7.6 % ± 3.4 of the sequence reads. At the later time points it constituted between 5.2 % and 8.5 % in the plant diet groups and between 3.2 % and 13.6 % in the marine diet groups. A significant difference was seen in both the marine diet groups, where the abundance was significantly lower at 49 days post f.f. in comparison to 26 days post f.f. ($p < 0.05$). Phylum Cyanobacteria ranked as the fifth highest before f.f. and constituted 1.5 % ± 3.4 of the entire reads. No significant differences were seen between the diet groups ($p > 0.05$), but the abundance was significantly higher in the plant groups 26 days post f.f. compared to 1 day before f.f. ($p < 0.05$). Further, the abundance was significantly higher in marine + pro and plant groups 49 days post f.f. relative to before f.f. ($p < 0.05$). Spirochaetes was the sixth most abundant phylum before f.f. and constituted 1.1 % ± 3.2 of the entire reads. However, it was not within the 10 most abundant phyla 26 and 49 days post f.f. The abundance for Spirochaetes was significantly lower for plant diet groups 26 days post f.f. ($p < 0.001$) and for all groups 49 days post f.f. ($p < 0.0001$). For all diet groups together, the mean abundance of Spirochaetes decreased to 0.04 % ± 0.003 at 26 days post f.f. and down to 0.004 % ± 0.0002 at 49 days post f.f. The remaining four phyla Planctomycetes, Tenericutes, Fusobacteria and Verrucomicrobia constituted less than 1 % of the total bacterial community each before f.f. However, for the sampling points after f.f. Planctomycetes and Fusobacteria had an average abundance above 1 % and will thus be included in the statistical analysis. The abundance of Planctomycetes increased significantly 26 days post f.f. for all four diet groups relative to before f.f. and had a mean abundance of 2.0 % ± 3.2 for the groups together ($p <$

0.001). No difference was seen 49 days post f.f. ($p > 0.05$). For phylum Fusobacteria the abundance increased significantly for all diet groups after first feeding and was significantly higher 26 and 49 days post f.f. ($p < 0.05$). The average abundance for the four diet groups was then 0.7 % ± 0.9 26 days post f.f. and 1.3 % ± 2.6 at 49 days post f.f. The remaining phyla SAR406, Acidobacteria, TM6, TM7 and Verrucomicrobia had all abundances below 1 %.

3.6. Microbial community profiling - genus level

The 15 most abundant genera at each sampling date are shown in Fig. 4, a-c. Specific details about the abundance of each genus in the single fish can be obtained in supplementary material S1-S3. The total abundance of these genera relative to the total amount of reads within a given sampling was 75.0 % for 1 day before f.f. and 69.7 % and 63.4 % for the samplings 26 and 49 days post f.f., respectively. As shown on the figures some sequences could only be classified to family- or class level. Overall there was no effect of the *P. acidilactici* probiont at genus level ($p > 0.05$), except on genus *Streptophyta*, which had a significantly higher relative abundance in the marine + pro diet group relative to the marine group at both samplings post f.f. ($p < 0.05$). In continuation to that the amount of reads taxonomically belonging to genus *Pediococcus* in the + pro diet groups after f.f. was low and constituted overall only (mean ± SD) 0.005 % ± 0.004 and 0.034 % ± 0.08 of the reads at the samplings 26 and 49 days post f.f., respectively.

The dominant genus before first feeding was *Sediminibacterium* belonging to phylum Bacteroidetes and constituted 42.7 % of the entire reads. The relative abundance was significantly lower for the plant diet groups 26 days after f.f. and was decreased to below 1 % 49 days post f.f. ($p < 0.05$). The relative abundance was 24.0 % and 31.9 % 26 days post f.f. for the marine + pro and marine groups, but significantly decreased to 4.8 % and 2.3 %, respectively after 49 days post f.f. The relative abundance of *Sediminibacterium* in the marine + pro diet group at 49 days post f.f. was significantly

higher than both plant diet groups ($p < 0.05$). The higher abundance of the Firmicutes in the plant diet groups relative to the marine diet groups was due to a significantly higher proportion of the genera *Streptococcus*, *Leuconostoc* and *Weissella* in the plant diet groups. These three genera had overall a higher relative abundance in the plant diet groups of between 3.2 and 6.1 fold at the samplings post f.f. ($p < 0.05$). Dietary dependencies were also seen for phylum Proteobacteria. A significantly higher abundance of bacteria belonging to the orders Burkholderiales and Roseateles from the β-proteobacteria class was seen for the plant diet groups 26 days post f.f. ($p < 0.05$). The family *Oxalobacteraceae* (order Burkholderiales) were also present in significantly higher abundances (5.4 fold) within both plant diets in comparison to the marine diets ($p < 0.05$). At the latest time-point a significantly higher abundance of bacteria from the families *Thiotrichaceae* and *Coxiellaceae* from the γ-proteobacteria class was seen in the marine diet group relative to the remaining groups ($p < 0.05$).

3.7. Multivariate analysis of bacterial communities

Multivariate analysis showed a relation between ontogeny, diet, and the composition of fish microbiota. Some of the genera explaining the highest degree of variation were low relative abundance genera. Before first feeding the PCA analysis of the microbiota revealed a fairly close plotting of individual fish except for a few outliers (Fig. 5a). The components 1 and 2 explained together 43.52 % of the variation in the factor analysis. The taxonomic mapping explaining the highest degree of variation belonged to Firmicutes (Ruminococcaceae, Clostridiales, Lachnospiraceae and *Staphylococcus*), Bacteroidetes (*Prevotella*, *Bacteriodales*, *Sediminibacerium*, Sphingobacteriales) and Actinobacteria (*Rhodococcus*, *Corynebacterium*). At 26 days post f.f. the fish clustered into two distinct groups dependent on diet type (Fig. 5b). Here, the bacteria explaining the highest degree of variation belonged almost all to phylum Proteobacteria (*Delftia*,

Enterobacteriaceae, *Stenotropomonas*, *Paucibacter*, Burkholderiales, Phyllobacteriaceae, *Mesorhizobium*, *Bradyrhizobium* and *Afipia*) and Bacteroidetes (*Sediminibacterium*). Component 1 and 2 explained together 50.23 % of the variation in the dataset. The last sampling point 49 days post f.f. showed a lower level of clustering between the marine- and plant diets and components 1 and 2 explained together 35.47 % of the variation (Fig. 5c). At this sampling point all bacteria explaining the highest degree of variation belonged to Proteobacteria (Rhodobacteraceae, *Shinella*, *Paracoccus*, *Rhizobium*, Coxiellaceae, Enterobacteriaceae, *Escherichia*, *Citrobacter*, *Shigella* and *Yersinia*). Despite the lower degree of clustering at this sampling point, individual fish appeared to be more widely distributed on the axis as in comparison to before f.f.

3.8. Intestinal gene regulation

The intestinal transcription level of a panel of immune related genes was measured from the fish before and after f.f. (Fig. 6, a-b). Except for the iNOS, C3 and membrane bound IgM gene the remaining examined immune genes turned out to change significantly in transcription level. Overall the regulation was mostly linked to the ontogenic state of the fish rather than the type of diet. Except for minor differences of between 1.7 and 2.4 folds within the plant diets (+/- probiotics) for CD8, MBL 2 and FOXP3b at 49 days post f.f. there was no significant difference in the transcription level between the diet types ($p < 0.05$). Further, for all genes except for complement factor C5 the transcription level was significantly lower (higher C_t-value) before f.f. compared to after f.f. ($p < 0.05$). The difference was highest for the genes CD8, IgT, MBL 1 and IL-1β, which all had a higher mean transcription level of at least 48 fold after f.f. compared to before f.f.

4. Discussion

In the present study, the microbiota and immune system of the gut was examined in rainbow trout during the window around first feeding. This was performed in order to study how the ontogenic shift from yolk sac based feeding towards external feed intake of different diets influences the bacterial community and the immune system in the intestine of the fry. To our knowledge this is the first study using high-throughput sequencing for examination of the gut microbiota in rainbow trout during the onset of first feeding. Previous studies on the salmonid fish concerning diet and gut microbiota have mainly been performed on larger fish that usually have been primed from the surroundings for a longer period than recently hatched fry (Desai et al., 2012; Navarrete et al., 2012; Navarrete et al., 2010; Pond et al., 2006). In the present study it was thus hypothesized that the shift from yolk sac based feeding towards external feeding would influence the gut microbiota.

A direct relationship between the ontogenic development and the intestinal microbiota in rainbow trout was demonstrated. By administering two different diet types to the fish it was further shown that the gut microbiota of rainbow trout is plastic and capable of being manipulated by the diet from first feeding (Wong et al., 2013). Rape seed oil and pea meal was evaluated in this study, since they are common replacers for fish oil and fish meal in fish feed used in aquaculture. In order to keep focus on those bacteria that were attached to the intestinal epithelium and those associated with the mucus rather than from the feed, fecal matter was squeezed out during the sampling of intestinal tissue. Interestingly, the amount of bacteria as measured by 16S rDNA qPCR less than doubled in the 26 days that followed first feeding and remained the same thereafter. In other words, quantitatively the bacterial population in the intestine of rainbow trout is well established at an early ontogenic stage, which in these experiments was equal to 19 days after hatch (1 day post f.f.). Further, the diversity of bacteria measured by the Shannon diversty index significantly increased after first feeding independently on the type of diet, showing that intake of feed and aging affects

the microbial diversity in the gut. In line with this, previous studies also indicated increasing bacterial diversity during the early life stages of coho salmon (*Oncorhynchus kisutch*) (Romero et al., 2006). Despite the observed plasticity in the bacterial composition during the period around first feeding it was evident that on the coarse taxonomic scale the microbiota was dominated by the four phyla Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria. From two earlier studies in larger rainbow trout and in line with the results from this study it is indicated that those four phyla constitute the 'core' microbiota in the gut of rainbow trout after first feeding regardless of whether the fish has initiated first feeding and whether the feed has a marine or a plant based origin (Desai et al., 2012; Navarrete et al., 2012). A clear pattern between the sampling points showed that the plant based diet favoured the presence of bacteria from the Firmicutes phylum of bacteria, whereas the marine based diet favoured presence of Proteobacteria. The same trend was seen in adult rainbow trout using plant based diets containing meal or protein concentrate from pea or soy (Desai et al., 2012). Thus it can be concluded that the intestinal microbiota in rainbow trout can be manipulated at several life stages.

When examining the microbiota at genus level, the low-diversity community before first feeding was caused by *Sediminibacterium*, which constituted almost 43% of all sequence reads. In previous studies the genus *Sediminibacterium* has been isolated from aquatic environments such as environmental water sample and sediments, suggesting that microorganisms from the rearing tank water colonise the intestines of fish well before first feeding (Kampfer et al., 2011; Ou and Yuan, 2008). Unfortunately no water samples were taken during this experiment, which might have shown the influence of the microbiota in the surrounding environment. The higher amount of Firmicutes in plant fed fish were due to a significantly higher presence of the genera *Weissella*, *Streptococcus* and *Leuconostoc*, which are all lactic acid bacteria. What has favoured the presence of these genera in the plant diet fed fish is not known, but earlier studies have shown that polyunsaturated fatty acids

depress the intestinal lactobacilli population in fish, which supports the present findings, where a significantly lower amount was found in the marine diet fed fish (Ringø, 1993). What functional impact these bacteria could have on the fish intestine is unknown, but potentially they may have beneficial effects on the immune system and could help protecting the fish against pathogenic invasion through the intestinal surface (Nayak, 2010; Salinas et al., 2008). In that regard it could be interesting to examine whether the intestine of fish fed plant based diets will have a different load of pathogenic bacteria after an experimental infection.

Despite earlier studies showing effects of the probiont *Pediococcus acidilactici* when applied to fish (Merrifield et al., 2011; Ferguson et al., 2010), only minor effects were seen in this study. A commercial dosage of *P. acidilactici* was used in the present experiment, compared to a 10^2-10^3 times higher concentration in previous studies, which could explain the discrepancy. The applied dose could therefore likely explain why only a low fraction of the sequence reads belonged to genus *Pediococcus*. However, it should also be kept in mind that other factors such as life stage and duration of the experiment could play a role. Worth considering for future studies would also be the route of probiotic administration. Recent studies have shown that alternative routes of administration of probionts like addition to the rearing water can protect rainbow trout from parasitic adhesion to the skin mucus (Carbajal-Gonzalez et al., 2013).

Multivariate analysis of the microbial communities revealed that a mix of the phyla Firmicutes, Bacteroidetes and Actinobacteria created the variation before first feeding, while Proteobacteria explained the main part of the variation after first feeding. It was mainly bacteria from the α- and β-Proteobacteria classes that explained the variation 26 days post first feeding, while this shifted towards proteobacterial from mainly the γ-class at 49 days. Both the figures 4 and 5 thus show that a succession in the communities occurred between all three sampling days.

There is currently a growing focus on gut microbiota in relation to its influence on parameters such as health status, metabolism and generally a wide range of biological processes (Semova et al., 2012; Navarrete et al., 2012; Rawls et al., 2004). In fish as well as in other organisms such as mice, immune competence develops in concert with the ontogenic development (Lindner et al., 2012; Torroba and Zapata, 2003; Zapata et al., 1997). The gene transcription data showed that the intestinal immune system had a much higher transcription level after first feeding and that minor effects were observed following probiotic treatment in the plant diet group. Particularly the markers for CD8, IgT, MBL1 and IL-1β were highly up-regulated following first feeding for all diet groups. These markers cover different parts of the immune system like those of the innate and adaptive and show thus that the immunological responses in connection to first feeding were broadly regulated. In this regard it should be asked whether this response is specifically caused by the present microbial environment or whether it is the opposite way around – namely that the gene transcription patterns dictates the composition of the microbial community. Since the differences found in transcription patterns were mainly related to the time points rather than type of diet it is assumed that ontogenic development might be regarded as the key player. However, it should also be considered that the increased amount of bacteria present in the intestine after first feeding directly could have led to a higher transcription of the immune apparatus, potentially mediated via interaction of the intestinal bacteria with e.g. toll-like receptors (Ingerslev et al., 2010). To support this, it is known from zebrafish that the transcription of innate immune genes like C3, C4 and SAA1 in germ-free fish are down-regulated in comparison to conventionally raised fish, thus showing that the immune system and gut microbiota are interrelated and cross-talking (Rawls et al., 2004). Minor up-regulations in transcription level of MBL2 and the T-cell markers CD8 and FOXP3b were seen for the plant probiotic diet group; however this was not reflected in the microbial pattern. Earlier findings using the exact same strain of *P. acidilactici*, but in a higher dose have reported changes in

immune parameters and the microbial community following administration to red tilapia (*Oreochromis niloticus*) (Ferguson et al., 2010). Previous papers have shown induction of enteritis and significant impact on the immune response in the gut of salmonid fish on plant-based diets (Marjara et al., 2012; Uran et al., 2009). These responses seem to being linked mainly to the proteins from the plants rather than the oil (Chikwati et al., 2012). Due to the transcriptional patterns in this study no sign of an inflammatory state was observed in the plant diet groups of fish relative to marine fed fish. We hypothesise that this could be related to the relative small size of the fish or in combination with the relative low content of pea protein in the plant diets, which only replaced 10% of the fish meal. Further, there are also some indications that rainbow trout are less responsive towards plant-based diets compared to e.g. Atlantic salmon, which could explain the results (*Salmo salar*) (Chikwati et al., 2012; Mansfield et al., 2010). Overall, significant changes in the gut microbiota at several taxonomic levels were seen in connection to first feeding independently of whether the diet had a marine or plant origin. Further, immune mechanisms may interact during this shift; however, the two different microbial profiles observed dependent on the diet type administered to the fish could not be explained directly by immune mechanisms.

Acknowledgements

The project Optifish is part of the Organic RDD programme, which is coordinated by International Centre for Research in Organic Food Systems, ICROFS. It is funded by The Danish AgriFish Agency, Ministry of Food, Agriculture and Fisheries (project journal no. 3405-10-OP-0128). Thanks should also be given to Anastasia Isbrand and Sophia Rasmussen at the National Veterinary Institute, Technical University of Denmark for excellent technical assistance during the laboratory work.

References

Arijo, S., Brunt, J., Chabrillon, M., az-Rosales, P., Austin, B., 2008. Subcellular components of Vibrio harveyi and probiotics induce immune responses in rainbow trout, *Oncorhynchus mykiss* (Walbaum), against V-harveyi. J. Fish Dis. 31, 579-590.

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A.C., Marquez, I., Girones, O., Muzquiz, J.L., 2007. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). Brit. J. Nutr. 97, 522-527.

Bates, J.M., Mittge, E., Kuhlman, J., Baden, K.N., Cheesman, S.E., Guillemin, K., 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Dev. Biol. 297, 374-386.

Bell, J.G., Farndale, B.M., Dick, J.R., Sargent, J.R., 1996. Modification of membrane fatty acid composition, eicosanoid production, and phospholipase A activity in Atlantic salmon (*Salmo salar*) gill and kidney by dietary lipid. Lipids 31, 1163-1171.

Caballero, M.J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M., Izquierdo, M.S., 2002. Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. Aquaculture 214, 253-271.

Carbajal-Gonzalez, M.T., Fregeneda-Grandes, J.M., Gonzalez-Palacios, C., aller-Gancedo, J.M., 2013. Adhesion to brown trout skin mucus, antagonism against cyst adhesion and pathogenicity to rainbow trout of some inhibitory bacteria against *Saprolegnia parasitica*. Dis. Aquat. Org. 104, 35-44.

Chikwati, E.M., Venold, F.F., Penn, M.H., Rohloff, J., Refstie, S., Guttvik, A., Hillestad, M., Krogdahl, A., 2012. Interaction of soyasaponins with plant ingredients in diets for Atlantic salmon, *Salmo salar* L. Brit. J. Nutr. 107, 1570-1590.

Dalsgaard, I. Madsen, L., 2000. Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. J. Fish Dis. 23, 199-209.

de Pablo, M.A. de Cienfuegos, G.A., 2000. Modulatory effects of dietary lipids on immune system functions. Immunol. Cell. Biol. 78, 31-39.

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G., Hill, J.E., 2012. Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 350, 134-142.

Falkow, S., 2006. Is persistent bacterial infection good for your health? Cell 124, 699-702.

Ferguson, R.M.W., Merrifield, D.L., Harper, G.M., Rawling, M.D., Mustafa, S., Picchietti, S., Balcazar, L., Davies, S.J., 2010. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). J. Appl. Microbiol. 109, 851-862.

Flint, H.J., Duncan, S.H., Scott, K.P., Louis, P., 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. Environmental Microbiology 9, 1101-1111.

Hamlin, H.J., von Herbing, I.H., Kling, L.J., 2000. Histological and morphological evaluations of the digestive tract and associated organs of haddock throughout post-hatching ontogeny. J. Fish Biol. 57, 716-732.

Hansen, G.H. Olafsen, J.A., 1999. Bacterial interactions in early life stages of marine cold water fish. Microb. Ecol. 38, 1-26.

Holt, R.A., Rohovec, J.S., Fryer, J.L. 1993. Bacterial coldwater disease. In: Inglis, V., Roberts, R.J. and Brombage, N.R. (Eds.), Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, UK, pp. 3-23.

Hovda, M.B., Lunestad, B.T., Fontanillas, R., Rosnes, J.T., 2007. Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). Aquaculture 272, 581-588.

Huber, I., Spanggaard, B., Appel, K.F., Rossen, L., Nielsen, T., Gram, L., 2004. Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). J. Appl. Microbiol. 96, 117-132.

Ingerslev, H.C., Ossum, C.G., Lindenstrom, T., Nielsen, M.E., 2010. Fibroblasts express immune relevant genes and are important sentinel cells during tissue damage in rainbow trout (*Oncorhynchus mykiss*). Plos One e9304, 1-9.

Jorgensen, L.V., Nemli, E., Heinecke, R.D., Raida, M.K., Buchmann, K., 2008. Immune-relevant genes expressed in rainbow trout following immunisation with a live vaccine against *Ichthyophthirius multifiliis*. Dis. Aquat. Org. 80, 189-197.

Kampfer, P., Lodders, N., Falsen, E., 2011. *Hydrotalea flava* gen. nov., sp. nov., a new member of the phylum *Bacteroidetes* and allocation of the genera *Chitinophaga*, *Sediminibacterium*, *Lacibacter*, *Flavihumibacter*, *Flavisolibacter*, *Niabella*, *Niastella*, *Segetibacter*, *Parasegetibacter*, *Terrimonas*, *Ferruginibacter*, *Filimonas* and *Hydrotalea* to the family *Chitinophagaceae* fam. nov. Int. J. Syst. Evol. Micr. 61, 518-523.

Kim, D.H., Brunt, J., Austin, B., 2007. Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). J. Appl. Microbiol. 102, 1654-1664.

Lindner, C., Wahl, B., Fohse, L., Suerbaum, S., Macpherson, A.J., Prinz, I., Pabst, O., 2012. Age, microbiota, and T cells shape diverse individual IgA repertoires in the intestine. J. Exp. Med. 209, 365-377.

Mansfield, G.S., Desai, A.R., Nilson, S.A., Van Kessel, A.G., Drew, M.D., Hill, J.E., 2010. Characterization of rainbow trout (Oncorhynchus mykiss) intestinal microbiota and inflammatory marker gene expression in a recirculating aquaculture system. Aquaculture 307, 95-104.

Mardis, E.R., 2008. Next-generation DNA sequencing methods. Annu. Rev. Genom. Hum. G. 9, 387-402.

Marjara, I.S., Chikwati, E.M., Valen, E.C., Krogdahl, A., Bakke, A.M., 2012. Transcriptional regulation of IL-17A and other inflammatory markers during the development of soybean meal-induced enteropathy in the distal intestine of Atlantic salmon (*Salmo salar* L.). Cytokine 60, 186-196.

Merrifield, D.L., Bradley, G., Harper, G.M., Baker, R.T.M., Munn, C.B., Davies, S.J., 2011. Assessment of the effects of vegetative and lyophilized *Pediococcus acidilactici* on growth, feed utilization, intestinal colonization and health parameters of rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquac. Nutr. 17, 73-79.

Mourente, G., Good, J.E., Thompson, K.D., Bell, J.G., 2007. Effects of partial substitution of dietary fish oil with blends of vegetable oils, on blood leucocyte fatty acid compositions, immune function and histology in European sea bass (*Dicentrarchus labrax* L.). Brit. J. Nutr. 98, 770-779.

Navarrete, P., Magne, F., Araneda, C., Fuentes, P., Barros, L., Opazo, R., Espejo, R., Romero, J., 2012. PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus mykiss*) gut microbiota reveals host-specific communities of active bacteria. Plos One e31335, 1-10.

Navarrete, P., Magne, F., Mardones, P., Riveros, M., Opazo, R., Suau, A., Pochart, P., Romero, J., 2010. Molecular analysis of intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*). Fems Microbiol Ecol 71, 148-156.

Nayak, S.K., 2010. Probiotics and immunity: A fish perspective. Fish Shellfish Immunol. 29, 2-14.

Ou, J.H. Yuan, H.L., 2008. *Sediminibacterium salmoneum* gen. nov., sp nov., a member of the phylum *Bacteroidetes* isolated from sediment of a eutrophic reservoir. Int. J. Syst. Evol. Micr. 58, 2191-2194.

Pedersen, R., Ingerslev, H.C., Sturek, M., Alloosh, M., Cirera, S., Christoffersen, B.O., Moesgaard, S.G., Larsen, N., Boye, M., 2013. Characterisation of gut microbiota in Ossabaw and Gottingen minipigs as models of obesity and metabolic syndrome. Plos One e56612, 1-10.

Pond, M.J., Stone, D.M., Alderman, D.J., 2006. Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 261, 194-203.

R Core Team, 2013. R: A language and environment for statistical computing. http://www. R-project. org

Rawls, J.F., Samuel, B.S., Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc. Natl. Acad. Sci. U. S. A. 101, 4596-4601.

Ringø, E., 1993. Does dietary linoleic acid affect intestinal microflora in Arctic charr, *Salvelinus alpinus* (L.)? Aquacult. Fish. Manage. 24, 133-135.

Ringo, E., Birkbeck, T.H., Munro, P.D., Vadstein, O., Hjelmeland, K., 1996. The effect of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L) larvae. J. Appl. Bacteriol. 81, 207-211.

Ringo, E. Gatesoupe, F.J., 1998. Lactic acid bacteria in fish: a review. Aquaculture 160, 177-203.

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., Rawls, J.F., 2011. Evidence for a core gut microbiota in the zebrafish. ISME. J. 5, 1595-1608.

Rombout, J.H.W.M., Abelli, L., Picchietti, S., Scapigliati, G., Kiron, V., 2011. Teleost intestinal immunology. Fish Shellfish Immunol. 31, 616-626.

Romero, J. Navarrete, P., 2006. 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). Microb. Ecol. 51, 422-430.

Salinas, I., Cuesta, A., Esteban, M.A., Meseguer, J., 2005. Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. Fish Shellfish Immunol. 19, 67-77.

Salinas, I., Myklebust, R., Esteban, M.A., Olsen, R.E., Meseguer, J., Ringo, E., 2008. In vitro studies of *Lactobacillus delbrueckii* subsp *lactis* in Atlantic salmon (*Salmo salar* L.) foregut: Tissue responses and evidence of protection against *Aeromonas salmonicida* subsp *salmonicida* epithelial damage. Vet Microbiol. 128, 167-177.

Semova, I., Carten, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S.A., Rawls, J.F., 2012. Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe 12, 277-288.

Shannon, C.E., 1948. A Mathematical Theory of Communication. Bell System Technical Journal 27, 623-656.

Silva, F.C.D., Nicoli, J.R., Zambonino-Infante, J.L., Kaushik, S., Gatesoupe, F.J., 2011. Influence of the diet on the microbial diversity of faecal and gastrointestinal contents in gilthead sea bream (*Sparus aurata*) and intestinal contents in goldfish (*Carassius auratus*). Fems Microbiol Ecol 78, 285-296.

Torroba, M. Zapata, A.G., 2003. Aging of the vertebrate immune system. Microsc. Res. Techniq. 62, 477-481.

Uran, P.A., Schrama, J.W., Jaafari, S., Baardsen, G., Rombout, J.H.W.M., Koppe, W., Verreth, J.A.J., 2009. Variation in commercial sources of soybean meal influences the severity of enteritis in Atlantic salmon (*Salmo salar* L.). Aquac. Nutr. 15, 492-499.

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F., Good, C., 2013. Aquacultured rainbow trout (*Oncorhynchus mykiss*)

possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. Applied and Environmental Microbiology 79, 4974-4984.

Zapata, A.G., Torroba, M., Varas, A., Jimenez, E. 1997. Immunity in fish larvae. In: Gudding, R., Lillehaug, A., Midtlyng, P. and Brown, F. (Eds.), Fish Vaccinology. Greenport, New York, pp. 23-32.

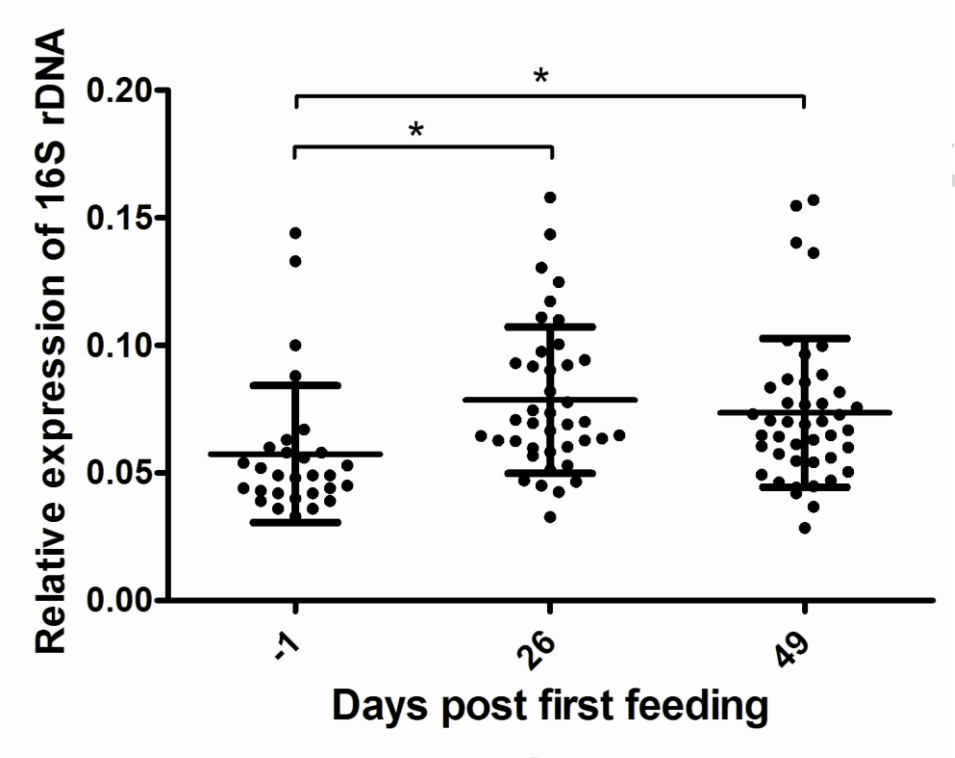

Fig. 1

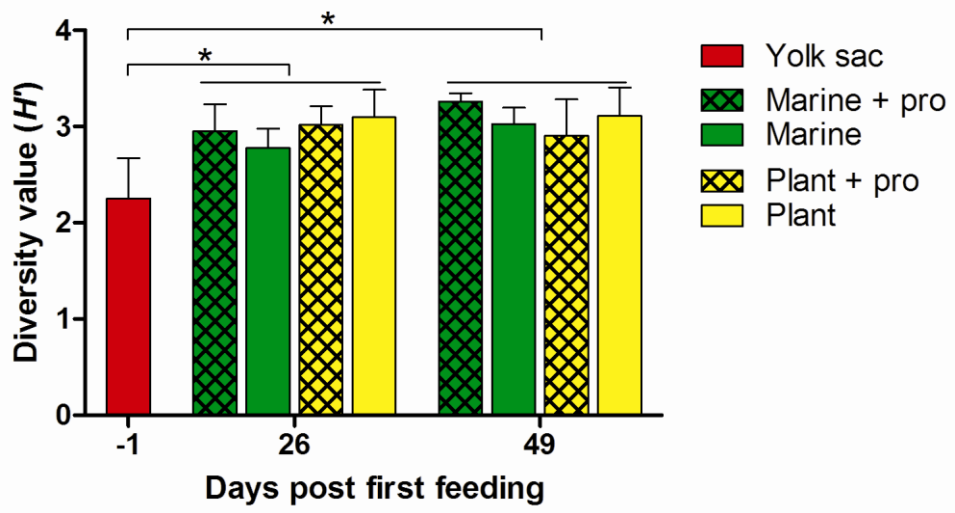

Fig. 2

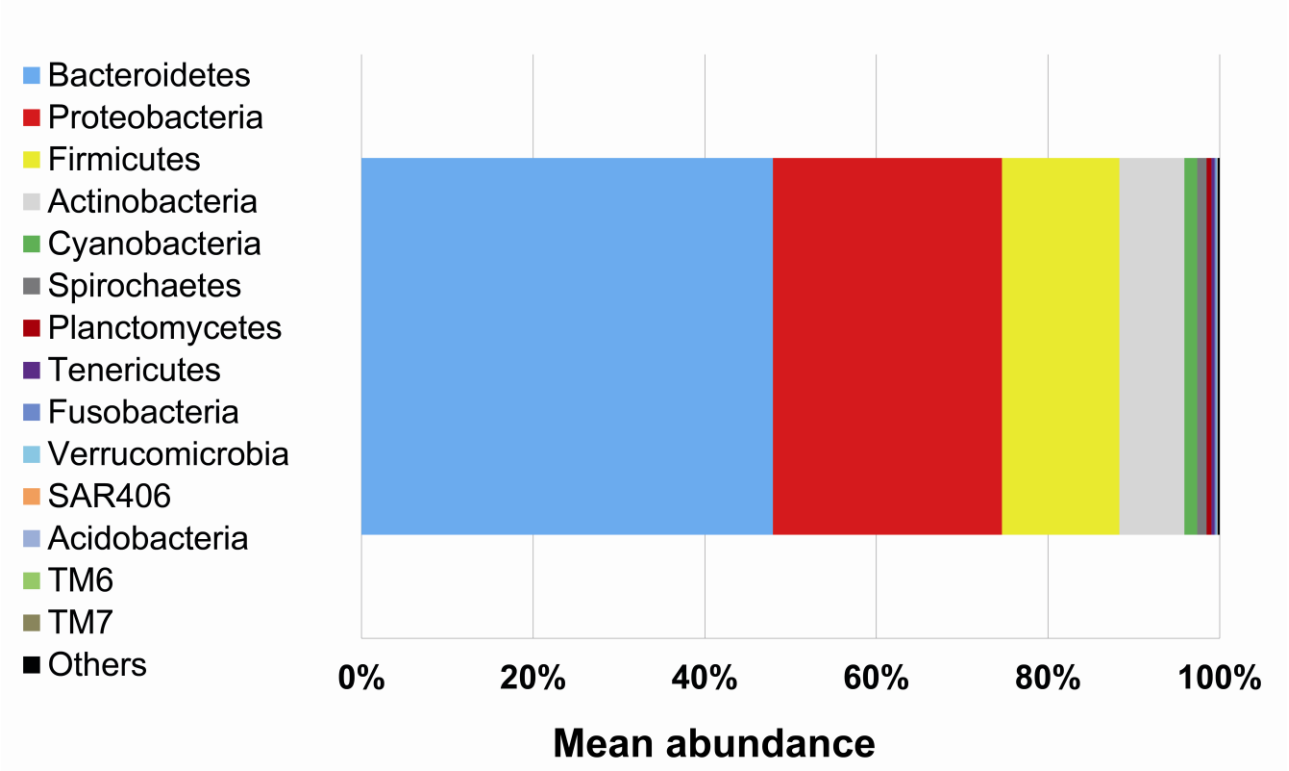

Fig. 3a

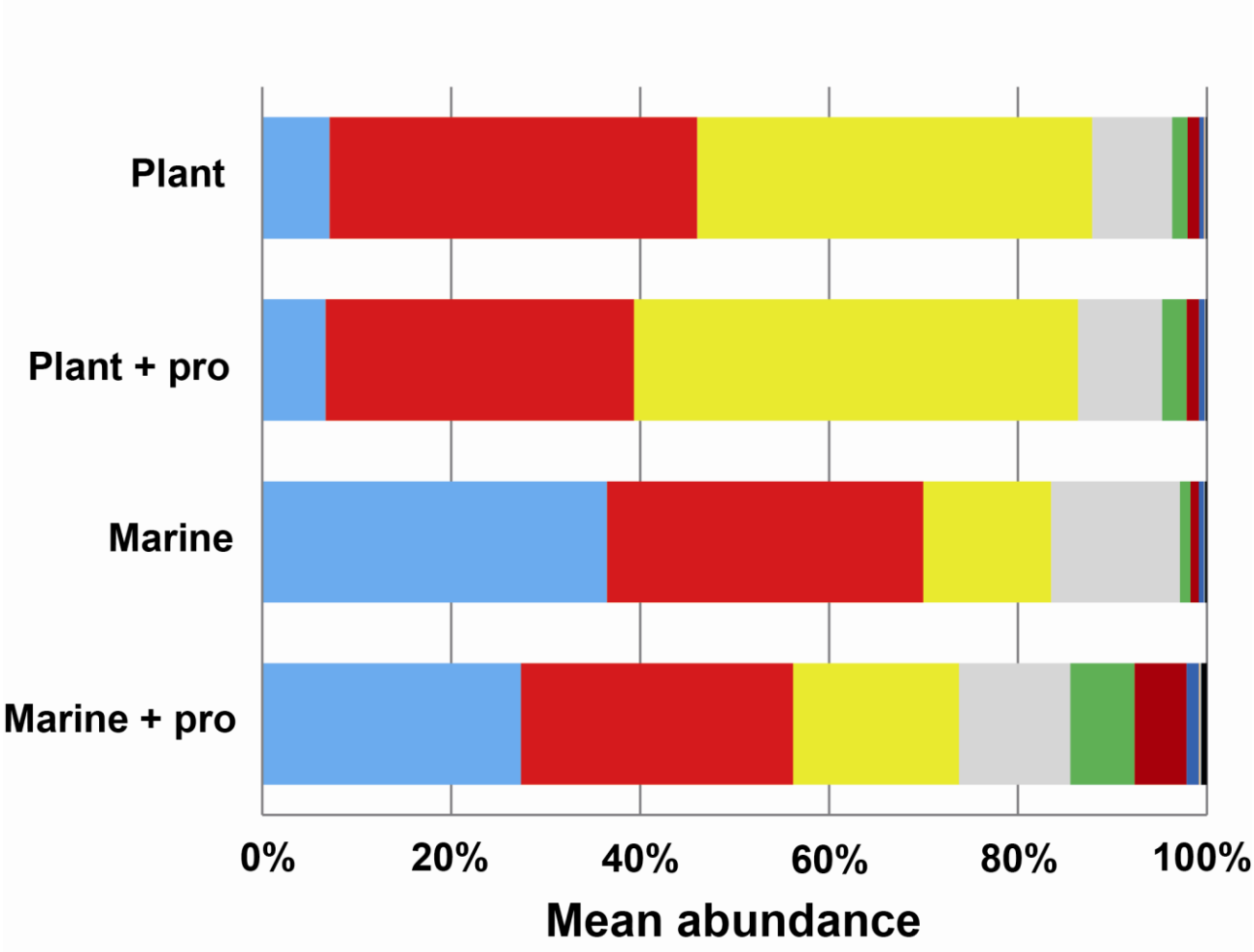

Fig. 3b

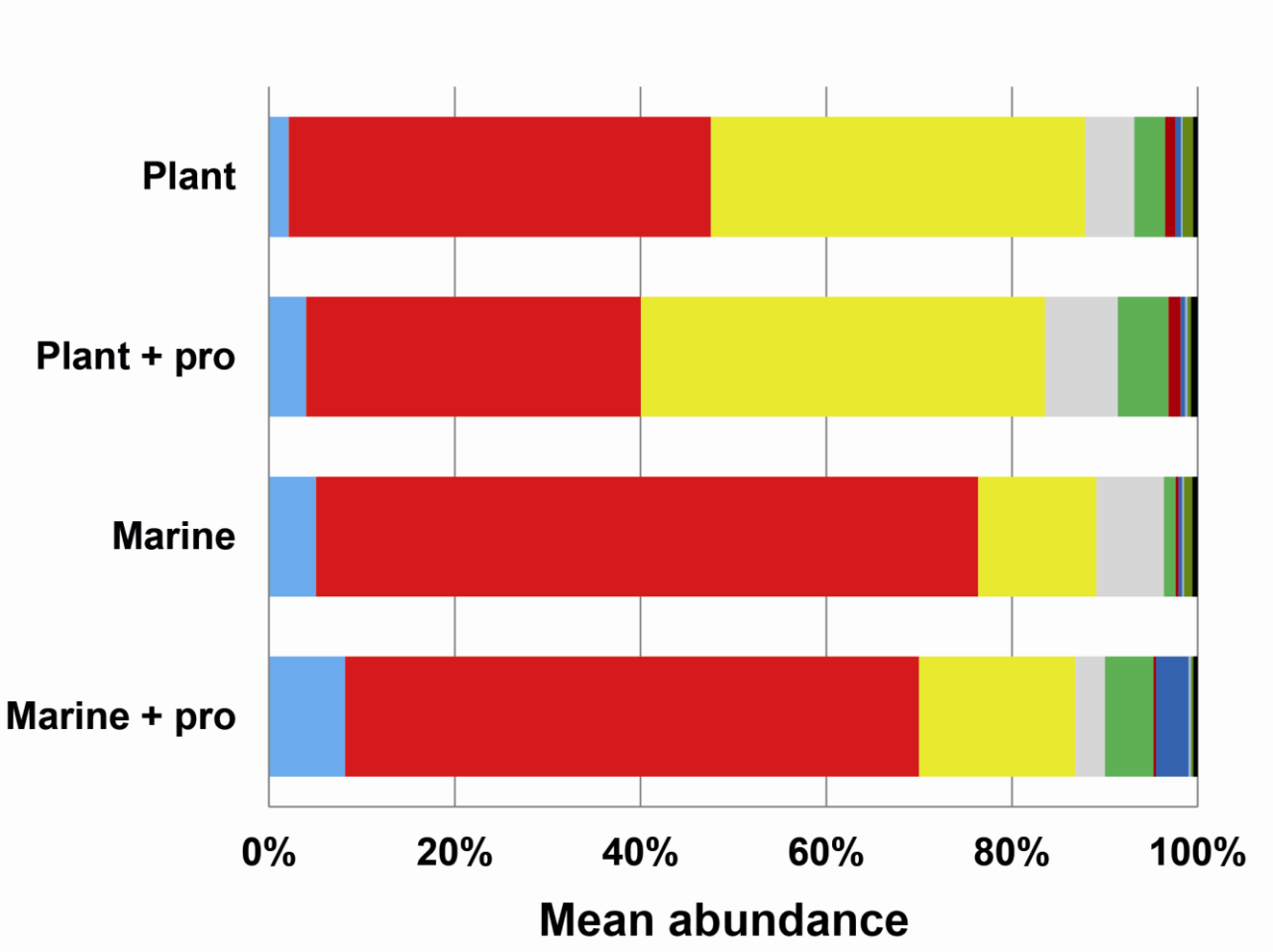

Fig. 3c

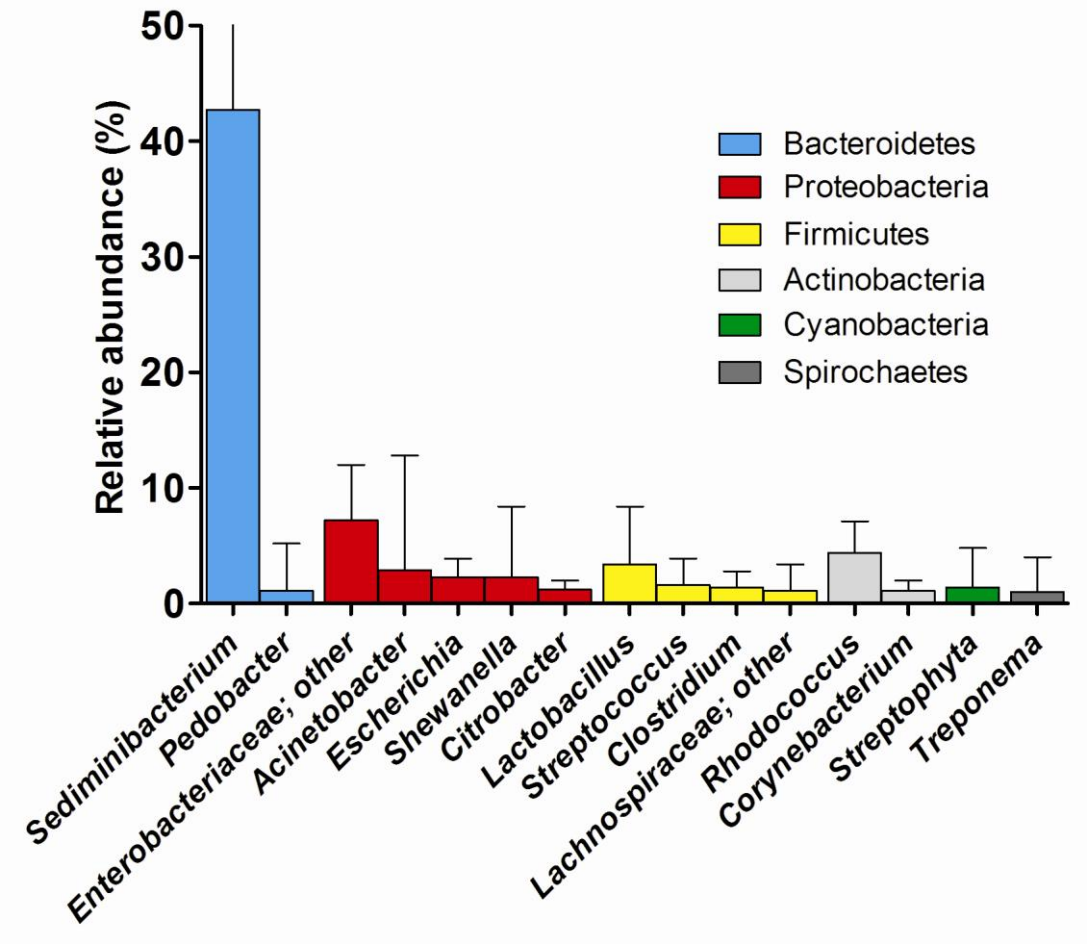

Fig. 4a

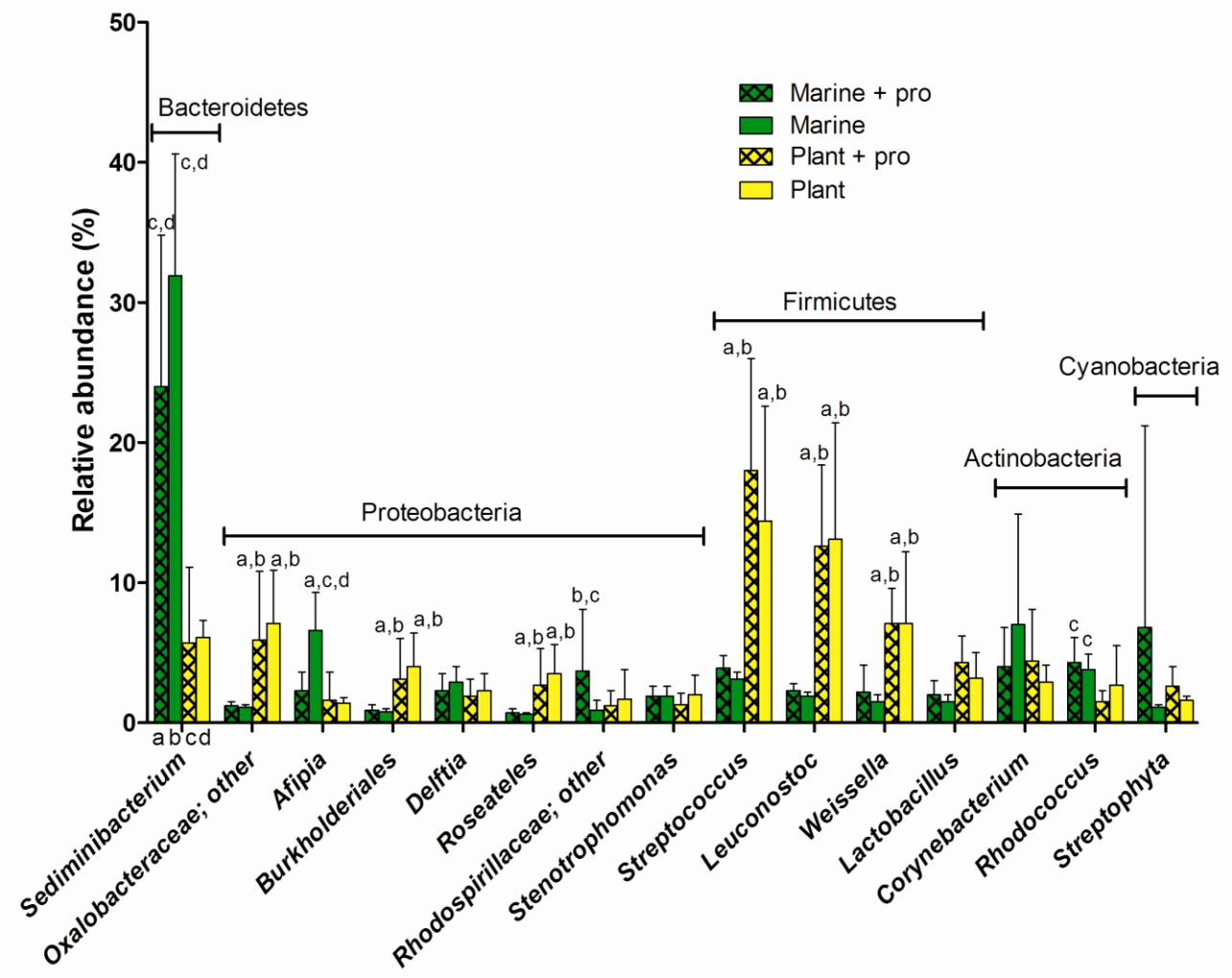

Fig. 4b

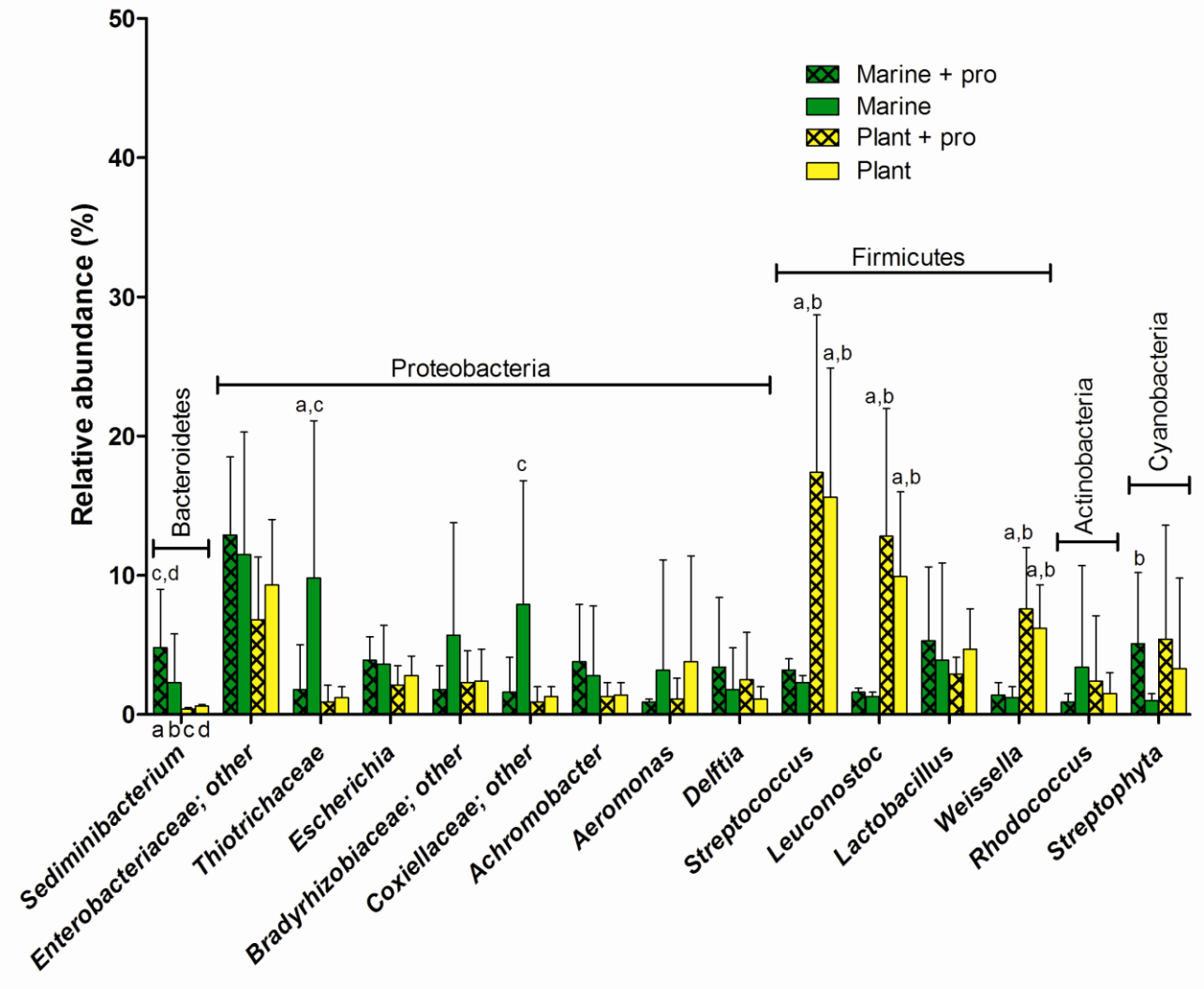

Fig. 4c

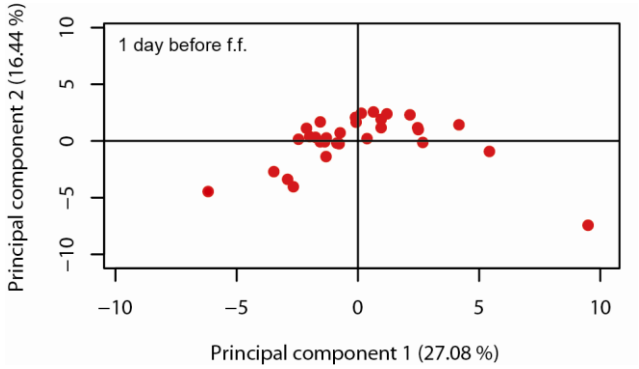

Fig. 5a

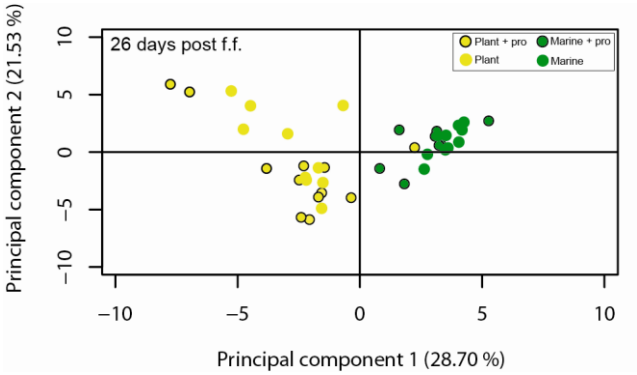

Fig. 5b

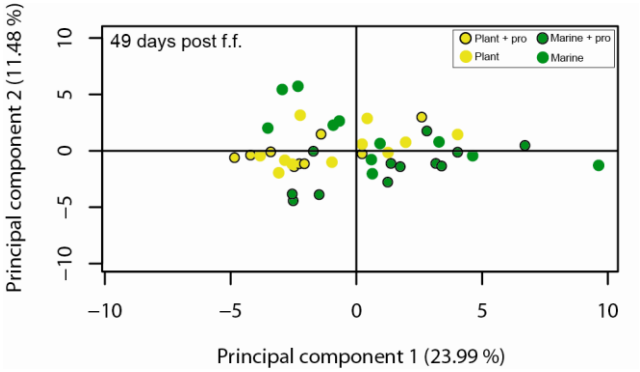

Fig. 5c

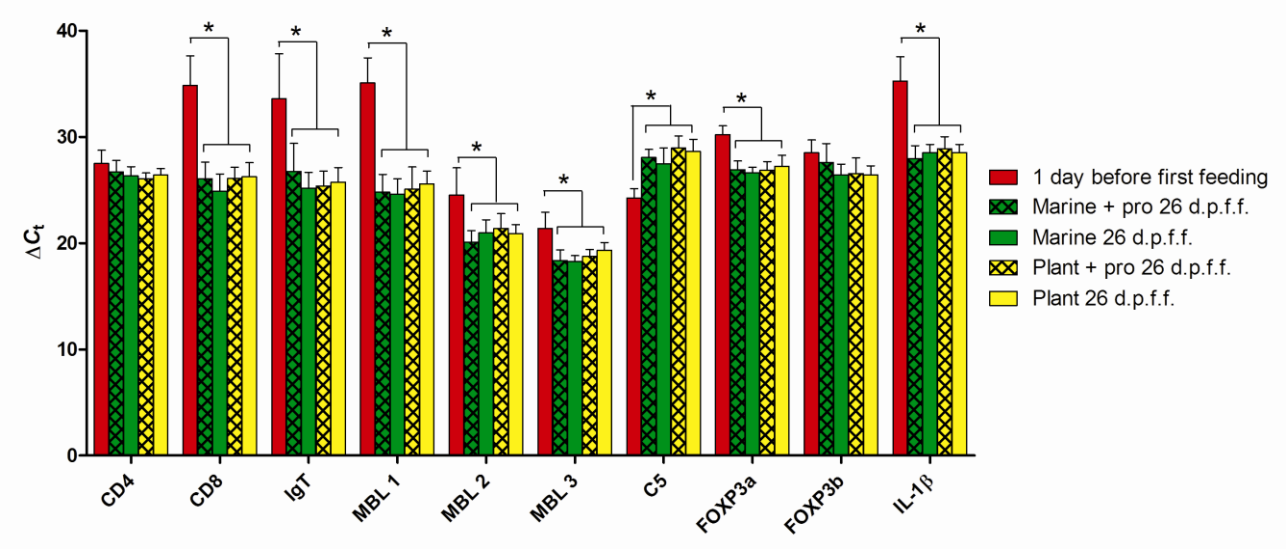

Fig. 6a

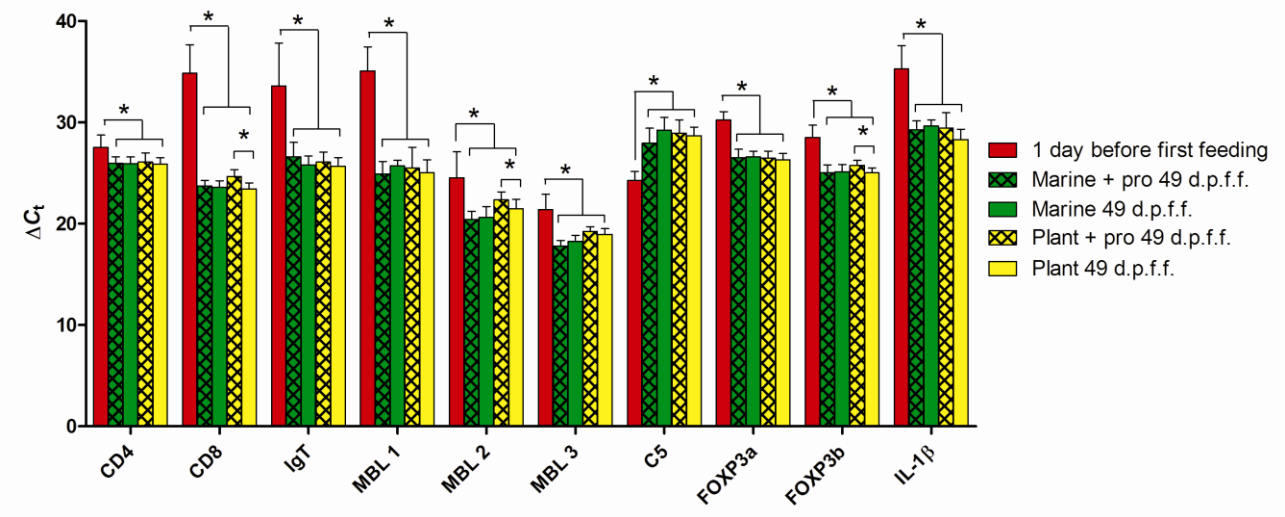

Fig. 6b

Table 1. List of primers and probes used for qRT-PCR.

Gene	Acc. no.	Product size (bp)	Primer sequence (5'-3')	Probe Sequence (5'-3')
Housekeeping genes				
28S	OMU34341	144	Fwd: TTCCCACTGTCCCTACCTACTATC Rev: CCTCCCACTTATTCTACACCTCTC	#
ELF-α	AF498320	63	Fwd: ACCCTCCTCTTGGTCGTTTC Rev: TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA
Beta-actin	AB196465	240	Fwd: ACATCAAGGAGAAGCTGTGCTAC Rev: TACGGATGTCCACGTCACAC	#
Innate immune genes				
MBL 1	EU118766	135	Fwd: ATGGCGATGCCCCGGTTG Rev: GGAAGTGTGAGGGGGAGG	TCTCAGCATTCTGCTGACCTCAC
MBL 2	EU118767	112	Fwd: ACTTTGGGTCTCTGTGTTC Rev: CACGAGGGCCAGGAATG	CAGAGGAGTGTGAATGCCGGGGT
MBL 3	EU118768	112	Fwd: ACTTTGGGGCTCTGTGTCT Rev: CACGAGGGCCAGGAGAA	TGGAGAAGTGTGAATGCCAGGGT
iNOS	AJ300555.1	109	Fwd: ACCAGAAGGAGGGTCACTT Rev: TGGGTGAGGGTGATGCCAA	ATGTGTGTGGGGGTGTGAACATGG
C3	AF271080	85	Fwd: ATTGGCCTGTCCAAAACACA Rev: GCTTCAGATCAAGGAAGAAGTTC	TGGAATCTGTGTGTCTGAACCCC
C5	AF349001	64	Fwd: TGGCAAGGACTTTTTCTGCT Rev: AGCACAGGTATCCAGGGTTG	CTGGCAGGGATTGCATCAAATC
IL-1β	AJ223954	91	Fwd: ACATTGCCAACCTCATCATCG Rev: TTGAGCAGGTCCTTGTCCTTG	CATGGAGAGGTTAAAGGGTGGC
Adaptive immune genes				
CD4	AF329700	73	Fwd: TCACCAGCAGACTGAGAGTCC	CCAATGAATGGCACAAACCAGAGAA

			Rev: AAGCTGACAATGCAGGTGAATC	
CD8	AF178054	74	Fwd: ACACCAATGACCACAACCATAGAG Rev: GGGTCCACCTTTCCCACTTT	ACCAGCTCTACAACTGCCAAGTCGTGC
FoxP3A	FM883710	80	Fwd: CTACAGGCACAGCCTGTCACTAGG Rev: GCTCCTCTGGCTCTTTAGTGG	CCAGAACCGAGGTGGAGTGTCACG
FoxP3B	FM883711	75	Fwd: TCCTGCCCCAGTACTCATCCC Rev: GCTCCTCTGGCTCTTTAGTGG	CTTGGCAGCAGATGGAGTGCCACG
Membrane bound IgM	S63348	72	Fwd: CTTGGCTTGTTGACGATGAG Rev: GGCTAGTGGTGTTGAATTGG	TGGAGAGAACGAGCAGTTCAGCA
Membrane bound IgT	AY870265	72	Fwd: AGCACCAGGGTGAAACCA Rev: GCGGTGGGTTCAGAGTCA	AGCAAGACGACCTCCAAAACAGAAC

= cyber green was used instead of a probe

Figure and table legends

Table 1 Sequence of primers and probes used for the RT-qPCR analysis.

Fig. 1. Amount of bacterial 16S rDNA in fish intestines at the different sampling days. The individual spots refer to the relative amount of 16S rDNA in a given individual sample after normalisation and bars indicate mean values ± SD. *n* = 29 (1 day before f.f.); *n* = 40 (26 days post f.f.); *n* = 43 (49 days post f.f.). * indicates significant difference ($p < 0.01$).

Fig. 2. Shannon diversity index (*H'*) for the fish at the different sampling days and for the different diet types. The bars show the mean *H'* value + SD. Red bars = 1 day before f.f.; Green bars = marine diets; yellow bars = plant diets. Gridded bars = diets containing *Pediococcus acidilactici*. *n* = 29 (1 day before f.f.); *n* = 40 (26 days post f.f.); *n* = 43 (49 days post f.f.). * indicates significant difference ($p < 0.0001$).

Fig. 3. Distribution of top-10 phyla at the different sampling days. **a** 1 day before f.f.; **b** 26 days post f.f.; **c** 49 days post f.f. Each colour shows the mean abundance (%) of a specific phylum. *n* = 31 (1 day before f.f.); *n* = 40 (26 days post f.f.); *n* = 43 (49 days post f.f.).

Fig. 4. Top-15 genera at the different sampling dates. **a:** 1 day before f.f. Each colour indicates a specific phylum. **b** and **c:** 26 and 44 days post f.f., respectively. Green bars = marine diets; yellow bars = plant diets. Gridded bars = diets containing *Pediococcus acidilactici*. The individual genera are grouped into phyla and shown in descending order within a given phylum. Some sequences could not be taxonomically determined to genus level, but only to family level and these taxon names are shown followed by '; other'. The bars show the mean relative abundance (%) of a given taxon + SD. Letters a-c above bars indicates statistical significant difference between diet treatments (Figs. **b** and **c**) ($p < 0.05$). *n* = 31 (1 day before f.f.); *n* = 40 (26 days post f.f.); *n* = 43 (49 days post f.f.).

Fig. 5. PCA plots of the bacterial communities in the intestines at the different sampling days. Each circle represents a specific fish. **a:** 1 day before f.f. Red spots = individual fish. **b** and **c:** 26 and 49 days post f.f., respectively. Green spots = marine diets; yellow spots = plant diets. Encircled spots indicate diets containing *Pediococcus acidilactici*. *n* = 31 (1 day before f.f.); *n* = 40 (26 days post f.f.); *n* = 43 (49 days post f.f.).

Fig. 6. Expression of immune-relatived genes measured by qRT-PCR. The figures show the expression of a given gene one day before f.f. relative to day 26 (**a**) or 49 (**b**) post first-feeding. Red bars = 1 day before f.f.; Green bars = marine diets; yellow bars = plant diets. Gridded bars = diets containing *Pediococcus acidilactici*. Only genes with a significant different expression pattern at one of the sampling dates are shown. The expression values are shown as mean ΔC_t-values + SD after normalization against the reference gene. * indicates significant difference ($p < 0.05$). *n* = 10 (1 day before f.f.); *n* = 40 (26 and 49 days post f.f.).

Highlights

We report how the gut microbiota in rainbow trout changes during first-feeding

The diet type significantly determined the gut microbiota after first-feeding

The bacterial diversity significantly increased after first-feeding

The ontogenic shift had a higher impact on the immune gene transcription than the type of diet